Conserv Genet (2010) 11:747–758
DOI 10.1007/s10592-009-9863-7

RESEARCH ARTICLE

Molecular and morphological assessment of Australia's most endangered snake, *Hoplocephalus bungaroides*, reveals two evolutionarily significant units for conservation

Joanna Sumner · Jonathan K. Webb · Richard Shine · J. Scott Keogh

Received: 14 December 2008 / Accepted: 16 February 2009 / Published online: 28 February 2009

Abstract The Broad-headed snake *Hoplocephalus bungaroides* is one of Australia's most endangered vertebrates. Extant populations of *H. bungaroides* are restricted to several geographically isolated reserves to the north, west, and south of Sydney. We analysed mitochondrial DNA from 184 specimens drawn from across the geographic range of the Broad-headed snake. Phylogenetic analysis demonstrated that *H. bungaroides* comprises two divergent mitochondrial lineages with a "northern" clade comprising populations west and north of Sydney and a "southern" clade comprising animals in Morton National Park. The two clades differ by an uncorrected genetic distance of 1.7%, which implies a divergence dating to approximately 755,000–850,000 years ago. We complemented our molecular data set with a detailed analysis of morphological variation both between and within the genetic clades. The two *H. bungaroides* genetic clades are morphologically indistinguishable and show little sexual dimorphism. Our results demonstrate that the populations north and south of this biogeographic split function as two distinct populations with no recent gene flow. There is no reason for separate taxonomic recognition of these two clades, but they do represent distinct evolutionarily significant units (ESUs) that require separate conservation management. In addition, within the northern ESU, populations from Royal National Park, Blue Mountains National Park, Wollemi National Park, and the Sydney Water Catchment supply areas should be considered as separate management units to conserve both evolutionary and ecological processes.

Keywords *Hoplocephalus bungaroides* · Phylogeography · Evolutionarily significant units · Conservation · Morphology

Introduction

Human land clearing activities have relegated populations of many threatened taxa to small geographically isolated habitat patches (Saunders et al. 1991). Species with poor dispersal ability or highly specialised habitat requirements may be unable to disperse between habitat patches, and consequently, they may face an increased risk of extinction (Goodman 1987; Hobbs and Yates 2003; McKinney 1997). Given limited funding and conflicts between conservation and other land-use activities, it is rarely possible to manage all habitat patches to conserve endangered species. The challenge for conservation biologists is to decide which populations or habitats to protect or manage in order to preserve both ecological and evolutionary processes (Frankel 1974). One solution to this problem is to use genetic techniques to identify evolutionarily significant units for conservation (ESU's), which in turn can aid in the management of the target species (Moritz 1994a, b; 2002). Molecular techniques can also help to identify management

J. Sumner · J. S. Keogh
School of Botany and Zoology, The Australian National University, Canberra, ACT 0200, Australia

Present Address:
J. Sumner (✉)
Museum Victoria, 11 Nicholson St., Carlton Gardens, Melbourne, VIC 3053, Australia
e-mail: jsumner@museum.vic.gov.au

J. K. Webb · R. Shine
School of Biological Sciences A08, University of Sydney, Sydney, NSW 2006, Australia

units (demographically distinct populations) that require management to maintain the viability of ESUs (Moritz 1994a, b, 2002). This approach, when combined with knowledge of the biology of the species in question, can allow land managers to identify the most important areas for management and conservation of the target species and their habitats.

The endangered Australian Broad-headed snake, *Hoplocephalus bungaroides*, is a medium-sized (to 90 cm long), brightly coloured, nocturnal, venomous snake that only occurs on sandstone rock formations within a 200 km radius of Sydney (Cogger 2000; Fig. 1). In the 1800s, Broad-headed snakes were common in the Sydney region (Krefft 1869), but in the last century they have declined dramatically due to urban development of ridge tops and the collection of sandstone rocks for landscaping urban gardens (Hersey 1980; Shine and Fitzgerald 1989). Today, the Broad-headed snake is locally extinct in the Sydney metropolitan area, and extant populations are restricted to a handful of geographically isolated national parks and water catchment reserves in the areas surrounding Sydney (Shine et al. 1998). Recent surveys suggest that the snake is locally extinct or rare within many national parks and reserves (Goldingay 1998; Newell and Goldingay 2005; Shine et al. 1998; Fig. 1). Extant populations are threatened by the removal of rocks for landscape gardening (Hersey 1980; Shine et al. 1998), the displacement and breakage of rocks caused by reptile collectors (Goldingay and Newell 2000), the collection of snakes for the illegal pet trade (Webb et al. 2002a), and the overgrowth of rock outcrops by encroaching vegetation (Pringle et al. 2003; Webb et al. 2005).

Despite its endangered status, the demography, ecology and behaviour of the Broad-headed snake is well known. Unlike other elapid snakes, Broad-headed snakes mature late (around 5 years), are long-lived, and females produce small clutches every second or third year (Webb et al. 2002b). These life history traits render populations of this species vulnerable to extinction, and the snake has disappeared from much of its former geographic range (Newell and Goldingay 2005; Shine et al. 1998). In Morton National Park, in the southern part of their range, Broad-headed snakes are restricted to a handful of disjunct plateaus, but long-term mark-recapture studies suggest that this population is small (<1,000 individuals, Webb et al. 2002a). During the cooler months, juvenile and adult Broad-headed snakes thermoregulate under thin rocks and inside crevices on rock outcrops (Webb and Shine 1998), a trait that makes them vulnerable to the removal of rocks for landscaping urban gardens (Shine et al. 1998). In summer, adult Broad-headed snakes disperse from rock outcrops to adjacent eucalypt forests, where they shelter inside tree hollows (Webb and Shine 1997a, b). Although adults can travel long distances during summer, most snakes return to the same rock outcrops where they were first captured (Webb and Shine 1997a, b). Mark-recapture studies suggest that Broad-headed snakes are poor dispersers, with most juveniles settling <1 km from their birth sites (Webb and Shine 1997b; Webb et al. 2002b). The high degree of adult philopatry and poor dispersal of juveniles could influence underlying genetic structuring among populations.

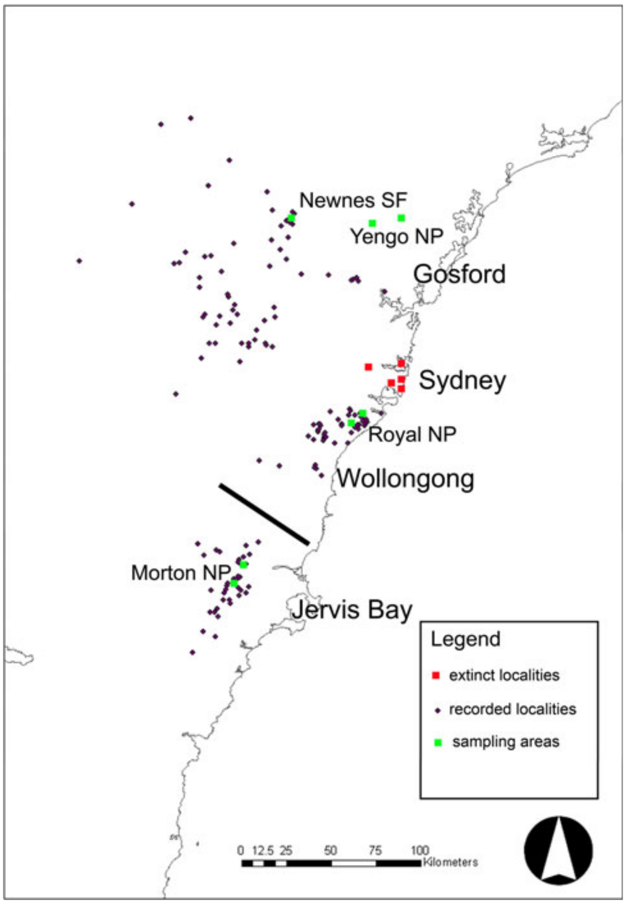

Fig. 1 Distribution map for *Hoplocephalus bungaroides* in the Sydney basin, in south eastern Australia. The *map* indicates all recorded localities from museum records, sites from which the snakes are known to be extinct and the sampling areas from which we have tissue samples used in this study. The *line* on the figure indicates the east-facing sandstone escarpment that is unsuitable for snake thermoregulation and which we consider to be a biogeographic break in *H. bungaroides*

In this study we evaluate phylogeographic structure among the remaining highly disjunct Broad-headed snake populations, and we complement our genetic data with morphological data taken from preserved specimens in museum collections. We use this information to make informed management recommendations to guide future conservation efforts of this critically endangered snake.

Materials and methods

Molecular data

We obtained a total of 184 tissue samples of *H. bungaroides* from sites throughout the current geographic range of the species including most of the reserves known to harbour populations (Fig. 1). We sampled populations from Morton National Park in the far south of the range, Royal National Park near Sydney, Yengo National Park north of Sydney, and Newnes State Forest northwest of Sydney. Where possible, we included samples from 3 to 4 individuals from each of these four regions to evaluate intra-population level variation (Table 1). We included a large number of samples from Morton National Park, which were obtained as part of a long-term mark-recapture study by two of us (J. K. W and R. S). We also included individuals captured during a recent survey of the distribution and abundance of this species and several samples were from captive individuals with reliable locality information. Because of the endangered status of this species, we used sloughed skins or scale clips to obtain tissue samples, and no animals were killed.

For each sample we targeted an approximately 900 bp DNA fragment of the mitochondrial genome which included the 3′ half of the ND4 gene and most of the tRNA cluster containing the histidine, serine and leucine tRNA genes. The target fragment was amplified using modified primers ND4 and Leu (Arévalo et al. 1994). This region was targeted because work at comparable taxonomic levels in numerous other squamate reptile groups has revealed useful levels of variability and because this gene was particularly useful in a similar study of phylogeographic structure in *Hoplocephalus stephensi* (Keogh et al. 2003). All laboratory procedures are as in Scott and Keogh (2000). Aligned sequences were translated into amino acid sequences using the vertebrate mitochondrial genetic code. No premature stop codons were observed, so we conclude that all sequences obtained are true mitochondrial copies.

The closely related elapid snake *Notechis scutatus* was used as an outgroup (Keogh et al. 1998, 2000) and we combined our data with the ND4 data for *H. bitorquatus* and *H. stephensi* from Keogh et al. (2003) to provide a comparison of species-level divergence within *Hoplocephalus*. We used parsimony and Bayesian approaches to analyse the data with PAUP* v4.0b10 (Swofford 2002) and MrBayes (v3.0b4; Huelsenbeck and Ronquist 2001), respectively. For the parsimony analyses we used TBR branch swapping and ran the parsimony analyses five times from random starting points and with random sequence addition to confirm that overall tree space was well searched. For the Bayesian analyses we allowed all parameters to be estimated from the data during the runs. We used the default value of four Markov chains per run and also ran the full analysis four times to make sure overall tree-space was well sampled and to avoid being trapped in local optima. We ran each analysis for a total of 5,000,000 generations and sampled the chain every 100 generations (standard MrBayes settings), resulting in 50,000 sampled trees. We discarded the first 10,000 trees and used the last 40,000 trees to estimate Bayesian posterior probabilities. We used 1,000 unweighted non-parametric parsimony bootstrap replicates and Bayesian posterior probabilities to assess branch support.

Morphological data

We collected extensive data on the morphology of *H. bungaroides* to test for any corroborating morphological differentiation between the mitochondrial clades. We examined all *H. bungaroides* specimens available in the following museum collections: Australian Museum (AM), Queensland Museum (QM), National Museum of Victoria (NMV), South Australian Museum (SAM), and the CSIRO Australian National Wildlife Collection (ANWC). After excluding any museum specimens with dubious or absent locality data, 61 specimens were available for analysis.

We collected data on all the external morphological characters traditionally used in elapid snake systematics. We measured scalation characteristics including nasal–preocular contact (present or absent); number of supralabial, infralabial, preocular, postocular, anterior temporal, and posterior temporal scales; number of dorsal scale rows one head length posterior to the neck, at mid-body, and one head length anterior to vent; and number of ventral and subcaudal scales. We measured snout-vent length (SVL), tail length, head length from the quadrate-articular projection at the rear of the jaw to the tip of the snout, head width at the widest part of the head, mouth length from posterior corner of the mouth and eye diameter.

After excluding characters that were largely invariant (nasal–preocular contact, number of supralabial, infralabial, preocular, postocular, anterior temporal, posterior temporal scales, dorsal scale rows), the remaining variables were natural log transformed prior to analysis to meet the assumptions of the statistical tests we employed. Each specimen was assigned to a genetic clade based on distribution. We then performed an analysis of variance (ANOVA) on each meristic character to test for differences between the genetic clades. For body size variables we used analysis of covariance (ANCOVA) against stable covariates with clade as the nominal variable. For tail length and head length we used SVL as the covariate and for head width, mouth length and eye diameter we used head length as the covariate. We then pooled all the data on adult specimens and performed the same analyses based on sex to test for sexual size dimorphism.

Table 1 Locality information for all samples used in the molecular data set for *Hoplocephaus bungaroides*

Sample	Location	Plateau	Site	Haplotype	Genbank no.
Northern clade					
Hb136	Newnes State Forest	N1	N1	C	FJ516665
Hb137	Newnes State Forest	N1	N1	D	FJ516666
Hb073	Putty	SC	Putty	C	FJ516607
Hb074	Putty	WS	Putty	E	FJ516608
Hb075	Putty	KW	Putty	C	FJ516609
Hb180	Royal National Park	Area 1	Area 1	F	FJ516703
Hb181	Royal National Park	Area 2	Area 2	C	FJ516704
Hb182	Royal National Park	Area 3	Area 3	C	FJ516705
Hb195	Yengo National Park	Y	MC	D	FJ516717
Southern clade					
Hb219	Bugong National Park	NB	NB	A	FJ516738
Hb211	Bugong National Park	SB	SB	A	FJ516731
Hb027	Morton National Park	MG	MG	B	FJ516562
Hb029	Morton National Park	MG	MG	B	FJ516564
Hb098	Morton National Park	MG	MG	B	FJ516629
Hb109	Morton National Park	MG	MG	A	FJ516640
Hb032	Morton National Park	MG	MG	A	FJ516567
Hb038	Morton National Park	MG	MG	A	FJ516573
Hb085	Morton National Park	MG	MG	A	FJ516619
Hb129	Morton National Park	MG	MG	A	FJ516660
Hb131	Morton National Park	MG	MG	A	FJ516662
Hb141	Morton National Park	MG	MG	A	FJ516668
Hb142	Morton National Park	MG	MG	A	FJ516669
Hb147	Morton National Park	MG	MG	A	FJ516674
Hb174	Morton National Park	MG	MG	A	FJ516700
Hb201	Morton National Park	MG	MG	A	FJ516723
Hb212	Morton National Park	MG	MG	A	FJ516732
Hb213	Morton National Park	MG	MG	A	FJ516733
Hb030	Morton National Park	NR	NR	A	FJ516565
Hb033	Morton National Park	NR	NR	A	FJ516568
Hb039	Morton National Park	NR	NR	A	FJ516574
Hb040	Morton National Park	NR	NR	A	FJ516575
Hb104	Morton National Park	NR	NR	A	FJ516635
Hb164	Morton National Park	NR	NR	A	FJ516690
Hb166	Morton National Park	NR	NR	A	FJ516692
Hb167	Morton National Park	NR	NR	A	FJ516693
Hb168	Morton National Park	NR	NR	A	FJ516694
Hb169	Morton National Park	NR	NR	A	FJ516695
Hb198	Morton National Park	NR	NR	A	FJ516720
Hb199	Morton National Park	NR	NR	A	FJ516721
Hb200	Morton National Park	NR	NR	A	FJ516722
Hb143	Morton National Park	YN	C	A	FJ516670
Hb192	Morton National Park	YN	C	A	FJ516715
Hb193	Morton National Park	YN	C	A	FJ516716
Hb209	Morton National Park	YN	C	A	FJ516729
Hb210	Morton National Park	YN	C	A	FJ516730

Table 1 continued

Sample	Location	Plateau	Site	Haplotype	Genbank no.
Hb035	Morton National Park	YN	Site 1	A	FJ516570
Hb043	Morton National Park	YN	Site 1	A	FJ516578
Hb044	Morton National Park	YN	Site 1	A	FJ516579
Hb050	Morton National Park	YN	Site 1	A	FJ516585
Hb052	Morton National Park	YN	Site 1	A	FJ516587
Hb054	Morton National Park	YN	Site 1	A	FJ516589
Hb055	Morton National Park	YN	Site 1	A	FJ516590
Hb063	Morton National Park	YN	Site 1	A	FJ516597
Hb064	Morton National Park	YN	Site 1	A	FJ516598
Hb065	Morton National Park	YN	Site 1	A	FJ516599
Hb066	Morton National Park	YN	Site 1	A	FJ516600
Hb067	Morton National Park	YN	Site 1	A	FJ516601
Hb068	Morton National Park	YN	Site 1	A	FJ516602
Hb071	Morton National Park	YN	Site 1	A	FJ516605
Hb080	Morton National Park	YN	Site 1	A	FJ516614
Hb084	Morton National Park	YN	Site 1	A	FJ516618
Hb091	Morton National Park	YN	Site 1	A	FJ516623
Hb092	Morton National Park	YN	Site 1	A	FJ516624
Hb093	Morton National Park	YN	Site 1	A	FJ516625
Hb099	Morton National Park	YN	Site 1	A	FJ516630
Hb100	Morton National Park	YN	Site 1	A	FJ516631
Hb101	Morton National Park	YN	Site 1	A	FJ516632
Hb110	Morton National Park	YN	Site 1	A	FJ516641
Hb111	Morton National Park	YN	Site 1	A	FJ516642
Hb113	Morton National Park	YN	Site 1	A	FJ516644
Hb114	Morton National Park	YN	Site 1	A	FJ516645
Hb116	Morton National Park	YN	Site 1	A	FJ516647
Hb121	Morton National Park	YN	Site 1	A	FJ516652
Hb125	Morton National Park	YN	Site 1	A	FJ516656
Hb133	Morton National Park	YN	Site 1	A	FJ516664
Hb154	Morton National Park	YN	Site 1	A	FJ516681
Hb156	Morton National Park	YN	Site 1	A	FJ516683
Hb158	Morton National Park	YN	Site 1	A	FJ516684
Hb173	Morton National Park	YN	Site 1	A	FJ516699
Hb183	Morton National Park	YN	Site 1	A	FJ516706
Hb184	Morton National Park	YN	Site 1	A	FJ516707
Hb187	Morton National Park	YN	Site 1	A	FJ516710
Hb188	Morton National Park	YN	Site 1	A	FJ516711
Hb191	Morton National Park	YN	Site 1	A	FJ516714
Hb005	Morton National Park	YN	Site 2	A	FJ516557
Hb010	Morton National Park	YN	Site 2	A	FJ516558
Hb011	Morton National Park	YN	Site 2	A	FJ516559
Hb036	Morton National Park	YN	Site 2	A	FJ516571
Hb041	Morton National Park	YN	Site 2	A	FJ516576
Hb042	Morton National Park	YN	Site 2	A	FJ516577
Hb045	Morton National Park	YN	Site 2	A	FJ516580
Hb046	Morton National Park	YN	Site 2	A	FJ516581

Table 1 continued

Sample	Location	Plateau	Site	Haplotype	Genbank no.
Hb047	Morton National Park	YN	Site 2	A	FJ516582
Hb048	Morton National Park	YN	Site 2	A	FJ516583
Hb051	Morton National Park	YN	Site 2	A	FJ516586
Hb053	Morton National Park	YN	Site 2	A	FJ516588
Hb056	Morton National Park	YN	Site 2	A	FJ516591
Hb058	Morton National Park	YN	Site 2	A	FJ516592
Hb059	Morton National Park	YN	Site 2	A	FJ516593
Hb060	Morton National Park	YN	Site 2	A	FJ516594
Hb061	Morton National Park	YN	Site 2	A	FJ516595
Hb069	Morton National Park	YN	Site 2	A	FJ516603
Hb070	Morton National Park	YN	Site 2	A	FJ516604
Hb072	Morton National Park	YN	Site 2	A	FJ516606
Hb081	Morton National Park	YN	Site 2	A	FJ516615
Hb083	Morton National Park	YN	Site 2	A	FJ516617
Hb089	Morton National Park	YN	Site 2	A	FJ516621
Hb090	Morton National Park	YN	Site 2	A	FJ516622
Hb095	Morton National Park	YN	Site 2	A	FJ516626
Hb096	Morton National Park	YN	Site 2	A	FJ516627
Hb097	Morton National Park	YN	Site 2	A	FJ516628
Hb105	Morton National Park	YN	Site 2	A	FJ516636
Hb108	Morton National Park	YN	Site 2	A	FJ516639
Hb112	Morton National Park	YN	Site 2	A	FJ516643
Hb117	Morton National Park	YN	Site 2	A	FJ516648
Hb120	Morton National Park	YN	Site 2	A	FJ516651
Hb123	Morton National Park	YN	Site 2	A	FJ516654
Hb124	Morton National Park	YN	Site 2	A	FJ516655
Hb128	Morton National Park	YN	Site 2	A	FJ516659
Hb130	Morton National Park	YN	Site 2	A	FJ516661
Hb140	Morton National Park	YN	Site 2	A	FJ516667
Hb144	Morton National Park	YN	Site 2	A	FJ516671
Hb145	Morton National Park	YN	Site 2	A	FJ516672
Hb146	Morton National Park	YN	Site 2	A	FJ516673
Hb148	Morton National Park	YN	Site 2	A	FJ516675
Hb149	Morton National Park	YN	Site 2	A	FJ516676
Hb150	Morton National Park	YN	Site 2	A	FJ516677
Hb151	Morton National Park	YN	Site 2	A	FJ516678
Hb152	Morton National Park	YN	Site 2	A	FJ516679
Hb153	Morton National Park	YN	Site 2	A	FJ516680
Hb155	Morton National Park	YN	Site 2	A	FJ516682
Hb178	Morton National Park	YN	Site 2	A	FJ516701
Hb179	Morton National Park	YN	Site 2	A	FJ516702
Hb185	Morton National Park	YN	Site 2	A	FJ516708
Hb189	Morton National Park	YN	Site 2	A	FJ516712
Hb190	Morton National Park	YN	Site 2	A	FJ516713
Hb196	Morton National Park	YN	Site 2	A	FJ516718
Hb197	Morton National Park	YN	Site 2	A	FJ516719
Hb207	Morton National Park	YN	Site 2	A	FJ516727

Table 1 continued

Sample	Location	Plateau	Site	Haplotype	Genbank no.
Hb013	Morton National Park	YN	Site 4	A	FJ516560
Hb014	Morton National Park	YN	Site 4	A	FJ516561
Hb062	Morton National Park	YN	Site 4	A	FJ516596
Hb102	Morton National Park	YN	Site 4	A	FJ516633
Hb107	Morton National Park	YN	Site 4	A	FJ516638
Hb122	Morton National Park	YN	Site 4	A	FJ516653
Hb132	Morton National Park	YN	Site 4	A	FJ516663
Hb159	Morton National Park	YN	Site 4	A	FJ516685
Hb160	Morton National Park	YN	Site 4	A	FJ516686
Hb161	Morton National Park	YN	Site 4	A	FJ516687
Hb162	Morton National Park	YN	Site 4	A	FJ516688
Hb163	Morton National Park	YN	Site 4	A	FJ516689
Hb165	Morton National Park	YN	Site 4	A	FJ516691
Hb170	Morton National Park	YN	Site 4	A	FJ516696
Hb186	Morton National Park	YN	Site 4	A	FJ516709
Hb214	Morton National Park	YN	Site 4	A	FJ516734
Hb215	Morton National Park	YN	Site 4	A	FJ516735
Hb034	Morton National Park	YN	Site 5	A	FJ516569
Hb037	Morton National Park	YN	Site 5	A	FJ516572
Hb082	Morton National Park	YN	Site 5	A	FJ516616
Hb103	Morton National Park	YN	Site 5	A	FJ516634
Hb115	Morton National Park	YN	Site 5	A	FJ516646
Hb118	Morton National Park	YN	Site 5	A	FJ516649
Hb119	Morton National Park	YN	Site 5	A	FJ516650
Hb126	Morton National Park	YN	Site 5	A	FJ516657
Hb127	Morton National Park	YN	Site 5	A	FJ516658
Hb204	Morton National Park	YN	Site 5	A	FJ516724
Hb206	Morton National Park	YN	Site 5	A	FJ516726
Hb208	Morton National Park	YN	Site 5	A	FJ516728
Hb001	Morton National Park	YN	Site 6	A	FJ516555
Hb002	Morton National Park	YN	Site 6	A	FJ516556
Hb028	Morton National Park	YN	Site 6	A	FJ516563
Hb031	Morton National Park	YN	Site 6	A	FJ516566
Hb049	Morton National Park	YN	Site 6	A	FJ516584
Hb086	Morton National Park	YN	Site 6	A	FJ516620
Hb106	Morton National Park	YN	Site 6	A	FJ516637
Hb076	Morton National Park	YNW	YNW	A	FJ516610
Hb077	Morton National Park	YNW	YNW	A	FJ516611
Hb078	Morton National Park	YNW	YNW	A	FJ516612
Hb079	Morton National Park	YNW	YNW	A	FJ516613
Hb217	Nowra	DG	AS1	A	FJ516736
Hb218	Nowra	DG	AS1	A	FJ516737
Hb205	Nowra	DG	AS2	A	FJ516725
Hb171	Nowra	DG	AS3	A	FJ516697
Hb172	Nowra	DG	AS3	A	FJ516698

For information on the *H. bitorquatus* and *H. stephensi* samples used, see Keogh et al. (2003). The samples are divided into the “northern” and “southern” *H. bungaroides* clades. Broad localities refer to sites noted in Fig. 1. Within these Broad localities, plateaus and sites within plateaus are listed. Haplotypes correspond to those shown in Fig. 2

Results

Molecular data

The edited alignment comprised 783 bp. Within the genus *Hoplocephalus*, 85 sites were variable and 77 of these were informative under parsimony. Both unweighted parsimony and Bayesian analyses produced the same topology and so in Fig. 2 we show an unweighted parsimony phylogram with both bootstrap values and Bayesian posterior probabilities. As we found in a previous study based on many fewer samples, *H. bungaroides* and *H. stephensi* are very closely related with an uncorrected genetic divergence of only 2.41–3.15% between them (Keogh et al. 2003). *Hoplocephalus bitorquatus* is more distantly related, with an uncorrected genetic distance of 6.91–7.36% between it and *H. bungaroides* and 7.29–7.77% between it and *H. stephensi*. *H. bungaroides* comprises two divergent clades that correspond geographically to two distinct regions surrounding Sydney. The "northern" clade comprises animals from Royal National Park, Newnes State Forest, Yengo National Park and Putty, whereas the "southern" clade comprises animals from Morton National Park, Bugong National Park and Nowra (Fig. 2). The two clades differ by an uncorrected genetic distance of 1.71–1.79%. Within these two clades, mitochondrial DNA variation is extremely low. Based on 175 sequences, the southern clade comprises just two haplotypes, which differ by a single base pair. The northern clade comprises fewer samples (9) but displays four closely related haplotypes which each differ by a single base pair (Fig. 2). Each of these divergent clades is supported by high bootstrap values (95–100%) and posterior probabilities of 100%. While support for the sister group relationship between the two clades is low, the morphological data strongly suggest monophyly (see below).

The commonly used rough mitochondrial DNA clock of approximately 2% divergence per million years (Avise 2004) dates the split between the two *H. bungaroides* clades to approximately 850,000 years ago. This is very similar to an estimate based on modern molecular genetic dating analyses for Australian elapids using multiple mtDNA and nDNA loci (Sanders et al. 2008). That study

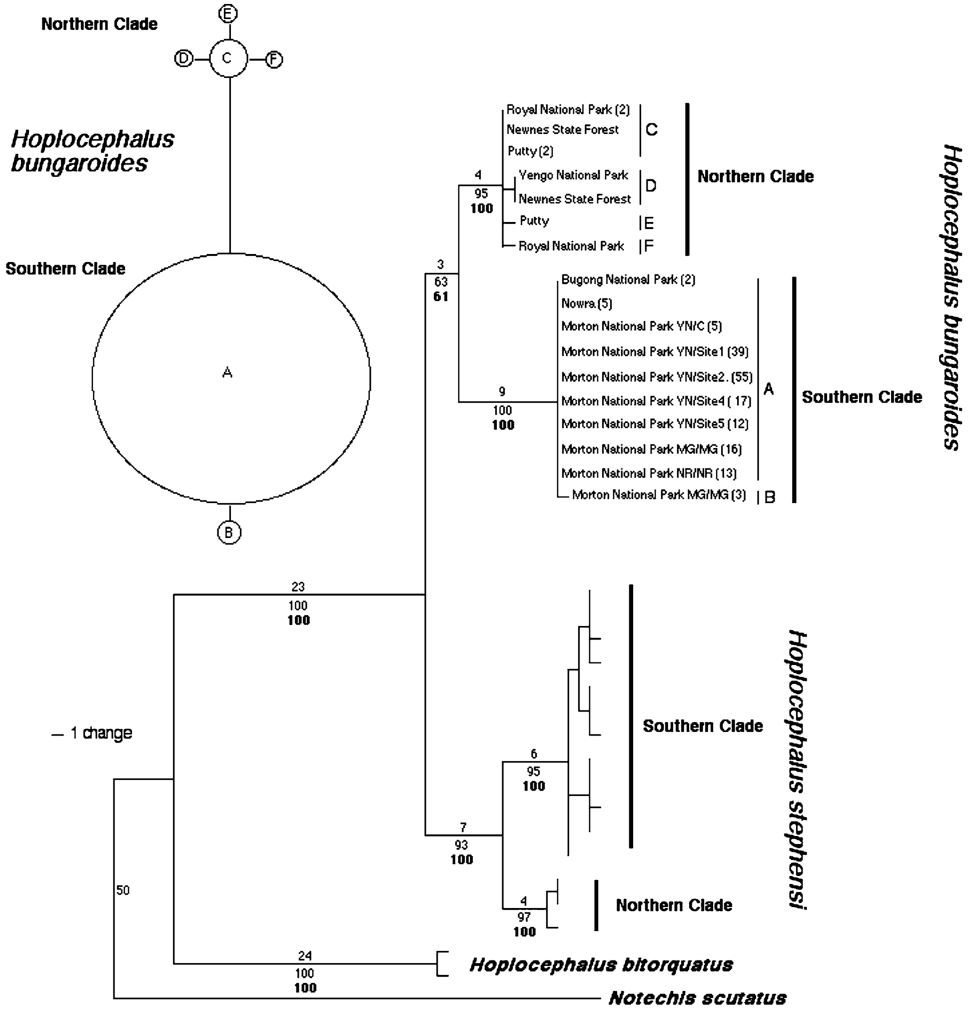

Fig. 2 Parsimony phylogram for the genus *Hoplocephalus* based on the mtDNA gene ND4 and associated tRNAs. Data for *H. stephensi* and *H. bitorquatus* from Keogh et al. (2003). For *H. bungaroides* sample sizes for each of the six haplotypes (A–F) are noted in *brackets* (see Table 1), and the northern and southern clades are identified. The *network diagram* illustrates relative haplotype frequency for *H. bungaroides*. The *numbers above* the branches represent uncorrected branch lengths and the *numbers below* the branch lengths represent parsimony bootstrap values and Bayesian posterior probabilities (*bold*)

Table 2 Summary statistics for body proportion measurements and tests for sexual size dimorphism (equivalent tests between the northern and southern clade showed no statistically significant differences)

	Male				Female					
	N	Mean	SD	Range	*N*	Mean	SD	Range	*F* value	*P* value
Number of ventral scales	31	210.29	5.066	201–222	28	214.214	4.23	207–223	10.564	0.0019
Number of subcaudal scales	31	54.387	2.813	49–59	26	49.865	2.52	46–56	40.09	0.0001
Snout-vent length (SVL)	31	387.355	116.715	183–590	28	456.357	162.049	196–685	1.098	0.3066
Tail length (SVL covariate)	30	67.5	22.682	35–109	26	72.704	24.132	30–110	7.69	0.0077
Head length (SVL covariate)	31	18.848	3.936	11.4–25.5	28	21.23	5.446	12.6–31.6	0.003	0.9553
Head width (head length covariate)	31	10.287	2.08	7.1–14.5	30	11.66	3.76	5.4–18.0	0.392	0.5335
Mouth length (head length covariate)	31	13.916	3.747	7.6–23.0	30	15.593	4.246	8.8–23.8	0.078	0.7804
Eye diameter (head length covariate)	31	2.361	0.367	1.7–3.1	30	2.437	0.46	1.6–3.8	2.577	0.1139

Raw data are presented, but statistical tests of sexual dimorphism were performed on log transformed data. ANOVA tests were performed on the number of ventral and subcaudal scales and snouth-vent length and one-factor ANCOVA tests were performed on the other variables where sex was the nominal variable and SVL, head length or head width was the covariate as indicated

dated the divergence between the clades comprising *N. scutatus* and *Hoplocephalus* (9.5% based on our mtDNA data) at approximately 4.2 mya which equates to approximately 2.25% divergence per million years. Extrapolating from this date to the divergence between the two *H. bungaroides* clades gives an estimate of approximately 755,000 years ago. However, it is worth noting that this mutation rate is specific to the portions of genes sampled and may vary considerably depending upon the rate of evolution of the sequenced region (e.g. Smith et al. 2007).

Morphological data

Hoplocephalus bungaroides populations show little morphological variation across their entire range. Scale characters associated with the head and the number of dorsal scale rows were largely invariant across the specimens examined. The range of variation in the number of ventral scales and the number of subcaudal scales was minimal and analyses showed no statistically significant differences between the two genetic clades in any of these characters. Similarly, analyses of the body size variables showed no significant differences in relative body proportion measurements between the two genetic clades. Given this homogeneity, we pooled the data to test for sexual size dimorphism across *H. bungaroides* as a whole. Like many snakes, females have significantly more ventral scales and fewer subcaudal scales than do males, with correspondingly longer tails in males (Table 2). Females were on average larger than males.

Discussion

Australia's most endangered snake, *H. bungaroides*, comprises two genetically divergent clades that correspond to distinct regions north and south of Sydney. This genetic divergence was not matched by morphological divergence. The two clades are morphologically homogeneous. Our results have important implications for understanding the evolutionary history of this species, and for future conservation management decisions.

The existence of two distinct genetic clades was unexpected. Molecular dating suggests that the two populations were isolated from each other during the late Pleistocene, approximately 755,000–850,000 years ago. During the Pleistocene, the climate in southeast Australia oscillated from warm, wet periods during interglacials to cold dry conditions during glacial periods (Byrne 2008). In the mid to late Pleistocene, significant changes in vegetation occurred during the glacial and interglacial periods (Dodson 1994; Wagstaff et al. 2001). These changes involved expansion and contraction of more mesic rainforest habitats. However, the available data suggest that there was little influence of Pleistocene climatic cycles on sclerophyll habitats (Byrne 2008). For example, populations of three animal species (two frogs and a glider) and a tree that occur in sclerophyll habitat along Australia's east coast show little evidence of phylogeographic structure (James and Moritz 2000; Brown et al. 2006; Jones et al. 2006; Burns et al. 2007).

The fact that Broad-headed snakes show significant geographic structuring suggests that Pleistocene climatic cycling may have restricted dispersal. Interestingly, the break between the southern and northern clade occurs in a narrow geologically distinct region where volcanic soils cover the sandstone plateau (Branagan and Packham 2000), resulting in few exposed sandstone rock outcrops that could act as 'stepping stones' for juvenile snake dispersal (Webb and Shine 1997b). In this region, a narrow east-facing sandstone escarpment runs west of Wollongong in the north through to Kangaroo Valley in the south, but the cliffs are swathed in tall closed forests that shade rocks and make them unsuitable for snake thermoregulation (Pringle et al. 2003). Previous field studies have shown that during the cooler months, Broad-headed snakes select sun-exposed rocks on west, north-west, or north-facing slopes or cliffs as diurnal thermoregulatory sites (Webb and Shine 1998). These sites allow snakes to attain high body temperatures at dusk, when the snakes ambush nocturnal lizards (Webb and Shine 1998). Both the Broad-headed snake and one of its main prey items, the velvet gecko, *Oedura lesueurii*, are rarely found under rocks on heavily timbered east-facing rock outcrops (Pringle et al. 2003). Hence, it is likely that this region formed a natural barrier to snake dispersal, particularly during colder glacial periods when the snakes would have required exposed areas on north or west facing exposed slopes for thermoregulation.

Our results reveal two evolutionarily significant units within the geographic range of the Broad-headed snake. Interestingly, genetic diversity was highest in the northern clade and lowest in the southern clade. This finding may reflect the greater geographic area covered by the northern clade. The presence of two distinct evolutionarily significant units (ESUs) within *H. bungaroides* has important implications for the management of this species. First, Broad-headed snakes are absent from several conservation reserves in the northern region where they were detected several decades ago (Newell and Goldingay 2005; Shine et al. 1998), which suggests that management is urgently needed to halt further declines. Second, extant Broad-headed snake populations are restricted to habitat fragments surrounded by a matrix unsuitable for snakes (urban areas, cleared lands, and pine plantations). Given the poor dispersal ability of this species (Webb and Shine 1997a), it is unlikely that snakes will disperse between adjacent conservation reserves in the future. For example, the population in Royal National Park is separated from western populations by urban areas and a major four-lane highway, both of which are potential barriers to snake dispersal (Foreman and Alexander 1998; Mader 1984; Roe et al. 2006). Each of these conservation reserves harbours viable populations of *H. bungaroides* and should be designated a separate management unit (MU) for conservation (Moritz 2002). Within the northern ESU, populations from Royal NP, Blue Mountains NP, Wollemi NP, and the Sydney Water Catchment supply areas also meet this criterion, and require careful management to prevent extinction of *H. bungaroides*. We also need more survey work to identify areas where Broad-headed snakes occur, particularly in the northern region that contains large expanses of protected habitat (e.g., Wollemi National Park).

The draft recovery plan for the Broad-headed snake lists captive breeding and return of captive-bred progeny to the wild as a possible conservation strategy (NSW Department of Environment and Climate Change, unpublished draft plan). Part of this plan involves releasing juveniles to areas where the species is currently extinct. Clearly, this strategy will need to carefully consider the genetic identity of adults used for breeding, and should ensure that captive bred progeny are released to the same region where the adults were obtained. However, unless factors causing the decline of *H. bungaroides* are ameliorated, the release of captive-born progeny to the wild is likely to fail (Caughley and Gunn 1996; Fischer and Lindenmayer 2000). Given that bush-rock removal has occurred throughout the northern geographic range of *H. bungaroides*, there is little point translocating animals to rock-denuded outcrops. Clearly, we need to develop methods to restore degraded habitats prior to translocating animals. One new method that is promising in this respect is the deployment of artificial concrete rocks that mimic the colour, shape, crevice structure, and thermal profile of natural rocks. These rocks were deployed in degraded rock outcrops, and were rapidly colonised by velvet geckos, and later, by Broad-headed snakes (Croak et al. 2009).

In conclusion, our study shows that extant Broad-headed snake populations display significant geographic structuring. This present day pattern probably resulted from changes in dispersal and/or habitat use that occurred in response to past climatic changes. Given that future changes in climate and vegetation are predicted to occur in the next century, we might expect future range contractions and local extinctions to occur in this sandstone-dependent species. Bioclimatic modelling to examine how this species responds to changes in climate, and how best to mitigate such effects, should be given a high priority if we are to conserve this iconic species.

Acknowledgments We thank Mark Fitzgerald and Jai Thomas for carrying out surveys for *H. bungaroides*, and Peter Harlow, David Pike and Simon Clulow for kindly providing tissue samples and locality data. Gary Daly and Ross Wellington provided expert advice on the distribution of Broad-headed snakes. We thank Les Mitchell, Phil Craven and Bruce Grey from the Department of Environment and Conservation (Nowra NPWS) for organising helicopter surveys and for their assistance in the field. Meagan Ewings (DEC Hurstville) helped organise surveys and assisted us during field work. We thank the curators and collection managers of all the Australian natural history museums who lent us the specimens in their care: Allen Greer and Ross Sadlier (Australian Museum), Jeanette Covacevich and Patrick Couper (Queensland Museum), John Coventry (National Museum of Victoria), Mark Hutchinson and Adrienne Edwards (South Australian Museum), and John Wombey (CSIRO Australian National Wildlife Collection). All research was carried out according to the University of Sydney Animal Care and Ethics Committee guidelines under a scientific licence from the New South Wales National Parks and Wildlife Service (Licence S10029). The research was supported financially by the Australian Research Council (grants to S. Keogh and R. Shine and a fellowship to J. Sumner), the University of Sydney (Sesqui Fellowship to J. Webb), the Australian Academy of Science (Margaret Middleton Award to J. Webb), and the Department of Environment and Climate Change NSW (grant to R. Shine).

References

Arévalo E, Davis SK, Sites J (1994) Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in Central Mexico. Syst Biol 43:387–418. doi:10.2307/2413675

Avise JC (2004) Molecular markers, natural history and evolution, 2nd edn. Sinauer Associates Inc., Sunderland

Branagan DF, Packham GH (2000) Field geology of New South Wales, 3rd edn. Department of Mineral Resources, Sydney

Brown M, Cooksley H, Carthew SM, Cooper SJB (2006) Conservation units and phylogeographic structure of an arboreal marsupial, the yellow-bellied glider (*Petaurus australis*). Aust J Zool 54:305–317. doi:10.1071/ZO06034

Burns EL, Eldridge MDB, Crayn DM, Houlden BA (2007) Low phylogeographic structure in a wide spread endangered Australian frog *Litoria aurea* (Anura: Hylidae). Conserv Genet 8:17–32. doi:10.1007/s10592-006-9143-8

Byrne M (2008) Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. Quat Sci Rev 27:2576–2585. doi:10.1016/j.quascirev.2008.08.032

Caughley G, Gunn A (1996) Conservation biology in theory and practise. Blackwell Science, Cambridge

Cogger HG (2000) Reptiles and amphibians of Australia, 6th edn. Reed New Holland, Sydney

Croak BM, Pike DA, Webb JK, Shine R (2009) Using artificial rocks to restore non-renewable shelter sites in human-degraded systems: colonization by fauna. Restor Ecol. doi:10.1111/j.1526-100X.2008.00476.x

Dodson JR (1994) Quaternary vegetation history. In: Groves RH (ed) Australian vegetation. Cambridge University Press, Cambridge, pp 37–56

Fischer J, Lindenmayer DB (2000) An assessment of the published results of animal relocations. Biol Conserv 96:1–11. doi:10.1016/S0006-3207(00)00048-3

Foreman RTT, Alexander LE (1998) Roads and their major ecological effects. Annu Rev Ecol Syst 29:207–231. doi:10.1146/annurev.ecolsys.29.1.207

Frankel OH (1974) Genetic conservation: our evolutionary responsibility. Genetics 78:53–65

Goldingay R (1998) Between a rock and a hard place: conserving the Broad-headed snake in Australia's oldest National Park. Proc Linn Soc N S W 120:1–10

Goldingay RL, Newell DA (2000) Experimental rock outcrops reveal continuing habitat disturbance for an endangered Australian snake. Conserv Biol 14:1908–1912. doi:10.1046/j.1523-1739.2000.99458.x

Goodman D (1987) The demography of chance extinction. In: Soule ME (ed) Viable populations for conservation. Cambridge University Press, Cambridge, pp 11–34

Hersey F (1980) Broad-headed snake, *Hoplocephalus bungaroides*. In: Haegl C (ed) Endangered animals of New South Wales. National Parks and Wildlife Service, Sydney, pp 38–40

Hobbs RJ, Yates CJ (2003) Turner review no. 7. Impacts of ecosystem fragmentation on plant populations: generalising the idiosyncratic. Aust J Bot 51:471–488. doi:10.1071/BT03037

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755. doi:10.1093/bioinformatics/17.8.754

James CH, Moritz C (2000) Intraspecific phylogeography in the sedge frog *Litoria fallax* (Hylidae) indicates pre-Pleistocene vicariance of an open forest species from eastern Australia. Mol Ecol 9:349–358. doi:10.1046/j.1365-294x.2000.00885.x

Jones ME, Shepherd M, Henry RJ, Delves A (2006) Chloroplast DNA variation and population structure in the widespread forest tree, *Eucalyptus grandis*. Conserv Genet 7:691–703. doi:10.1007/s10592-005-9104-7

Keogh JS, Shine R, Donnellan S (1998) Phylogenetic relationships of terrestrial Australo-Papuan elapid snakes based on cytochrome b and 16S rRNA sequences. Mol Phylo Evol 10:67–81. doi:10.1006/mpev.1997.0471

Keogh JS, Scott IAW, Scanlon JD (2000) Molecular phylogeny of viviparous Australian elapid snakes: affinities of *'Echiopsis' atriceps* (Storr, 1980) and *'Drysdalia' coronata* (Schlegel, 1837), with description of a new genus. J Zool 252:317–326. doi:10.1111/j.1469-7998.2000.tb00626 (Lond)

Keogh JS, Scott IAW, Fitzgerald M, Shine R (2003) Molecular phylogeny of the Australian venomous snake genus *Hoplocephalus* and conservation genetics of the threatened *H. stephensii*. Conserv Genet 4:57–65. doi:10.1023/A:1021823423944

Krefft G (1869) The snakes of Australia; an illustrated and descriptive catalogue of all the known species. T Richards, Govt. Printer, Sydney

Mader HJ (1984) Animal habitat isolation by roads and agricultural fields. Biol Conserv 29:81–96. doi:10.1016/0006-3207(84)90015-6

McKinney ML (1997) Extinction vulnerability and selectivity: combining ecological and paleontological views. Annu Rev Ecol Syst 28:495–516. doi:10.1146/annurev.ecolsys.28.1.495

Moritz C (1994a) Defining evolutionarily significant units for conservation. Trends Ecol Evol 9:373–375. doi:10.1016/0169-5347(94)90057-4

Moritz C (1994b) Applications of mitochondrial DNA analysis in conservation: critical review. Mol Ecol 3:401–411. doi:10.1111/j.1365-294X.1994.tb00080.x

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. Syst Biol 51:238–254. doi:10.1080/10635150252899752

Newell DA, Goldingay RL (2005) Distribution and habitat assessment of the Broad-headed snake *Hoplocephalus bungaroides*. Aust Zool 33:168–179

Pringle RM, Webb JK, Shine R (2003) Canopy structure, microclimate, and habitat selection by a nocturnal snake, *Hoplocephalus bungaroides*. Ecology 84:2668–2679. doi:10.1890/02-0482

Roe JH, Gibson J, Kingsbury BA (2006) Beyond the wetland border: estimating the impact of roads for two species of water snakes. Biol Conserv 130:161–168. doi:10.1016/j.biocon.2005.12.010

Sanders KL, Lee MSY, Leijs R, Foster R, Keogh JS (2008) Molecular phylogeny and divergence dates for Australasian elapids and sea snakes (Hydrophiinae): evidence from seven genes for rapid evolutionary radiations. J Evol Biol 21:882–895. doi:10.1111/j.1420-9101.2008.01525.x

Saunders DA, Hobbs RJ, Margules CR (1991) Biological consequences of ecosystem fragmentation: a review. Conserv Biol 5:18–32. doi:10.1111/j.1523-1739.1991.tb00384.x

Scott IAW, Keogh JS (2000) Conservation genetics of the endangered grassland earless dragon *Tympanocryptis pinguicolla* (Reptilia: Agamidae) in Southeastern Australia. Conserv Genet 1:357–363. doi:10.1023/A:1011542717349

Shine R, Fitzgerald M (1989) Conservation and reproduction of an endangered species: the Broad-headed snake, *Hoplocephalus bungaroides* (Elapidae). Aust Zool 25:65–67

Shine R, Webb JK, Fitzgerald M, Sumner J (1998) The impact of bush-rock removal on an endangered snake species, *Hoplocephalus bungaroides* (Serpentes, Elapidae). Wildl Res 25:285–295. doi:10.1071/WR97022

Smith SA, Sadlier RA, Bauer AM, Austin CC, Jackman T (2007) Molecular phylogeny of the scincid lizards of New Caledonia and adjacent areas: evidence for a single origin of the endemic skinks of Tasmantis. Mol Phylo Evol 43:1151–1166. doi: 10.1016/j.ympev.2007.02.007

Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland

Wagstaff BE, Kershaw AP, O'Sullivan PB, Harle KJ, Edwards J (2001) An Early to Middle Pleistocene palynological record from the volcanic crater of Pejark Marsh, Western Plains of Victoria, southeastern Australia. Quat Int 83–85:211–232. doi: 10.1016/S1040-6182(01)00041-6

Webb JK, Shine R (1997a) Out on a limb: conservation implications of tree-hollow use by a threatened snake species (*Hoplocephalus bungaroides*: Serpentes, Elapidae). Biol Conserv 81:21–33. doi: 10.1016/S0006-3207(96)00160-7

Webb JK, Shine R (1997b) A field study of spatial ecology and movements of a threatened snake species, *Hoplocephalus bungaroides*. Biol Conserv 82:203–217. doi:10.1016/S0006-3207(97)00032-3

Webb JK, Shine R (1998) Using thermal ecology to predict retreat-site selection by an endangered snake species. Biol Conserv 86:233–242. doi:10.1016/S0006-3207(97)00180-8

Webb JK, Brook BW, Shine R (2002a) Reptile collectors threaten Australia's most endangered snake, the Broad-headed snake *Hoplocephalus bungaroides*. Oryx 36:170–181. doi:10.1017/S0030605302000248

Webb JK, Brook BW, Shine R (2002b) What makes a species' vulnerable to extinction? Comparative life history traits of two sympatric snakes. Ecol Res 17:59–67. doi:10.1046/j.1440-1703.2002.00463.x

Webb JK, Pringle RM, Shine R (2005) Canopy removal restores habitat quality for an endangered snake in a fire suppressed landscape. Copeia 2005:893–899. doi:10.1643/0045-8511(2005)005[0894:CRRHQF]2.0.CO;2